EPA/OPP MICROBIOLOGY LABORATORY
ESC, Ft. Meade, MD

Standard Operating Procedure for Test Microbes for the AOAC Use-Dilution Method, AOAC Germicidal Spray Products Test, AOAC Confirmatory Tuberculocidal Test, AOAC Sporicidal Activity Test Method, and the Quantitative Suspension Test Method: Culture Initiation, Culture Maintenance and Quality Control

SOP Number: MB-02-03

Date Revised: 11-09-05

Initiated By: _____ Date: ___/___/___

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Effective Date: ___/___/___

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TABLE OF CONTENTS

1.0 SCOPE AND APPLICATION:

1.1 This protocol describes the procedures and practices used to initiate, confirm, and maintain the bacterial cultures used in disinfectant efficacy testing.

2.0 DEFINITIONS: None

3.0 HEALTH AND SAFETY:

3.1 All manipulations of the test organism are required to be performed in accordance with biosafety practices stipulated in SOP MB-01, Lab Biosafety.

4.0 CAUTIONS:

4.1 Use aseptic techniques to prevent contamination.

4.2 Media indicated in sections 10.1.1.3 and 10.1.1.4 for rehydrating lyophilized cultures are specified on the ATCC Product Information Sheet that accompanies each organism. Upon purchase of new organisms, verify that media requirements have not changed by checking the new ATCC Product Information Sheet.

5.0 INTERFERENCES:

5.1 Contamination of stock cultures will negatively impact disinfectant efficacy testing. It is critical to maintain the highest standards of good laboratory practices and aseptic technique during all manipulations and handling of stock cultures.

6.0 PERSONNEL QUALIFICATIONS:

6.1 Personnel are required to be knowledgeable about and to comply with the laboratory's culturing and disinfectant testing procedures. Documentation of training and familiarization with these procedures can be found in the training file for each employee.

7.0 SPECIAL APPARATUS AND MATERIALS:

7.1 Incubator with temperature at 37±1ºC

7.2 Incubator with temperature at 30±1ºC

7.3 *Pseudomonas aeruginosa* (ATCC 15442), *Staphylococcus aureus* (ATCC 6538), and *Bacillus subtilis* (ATCC 19659); ordered annually and received directly from ATCC; *Mycobacterium bovis* (BCG); ordered and received directly from Organon Teknika.

7.4 Selective media in Petri dishes: Cetrimide Agar (Cetrimide), Mannitol Salt Agar (MSA), and Middlebrook 7H9 agar (M7H9).

7.5 BBL Gram Stain Kit

7.6 BBL TB Quick Stain Kit

7.7 Vitek 32 System for the automated identification of microorganisms

7.8 Vitek 32 Identification Cards (GNI, GPI+, and BAC)

7.9 #18 Broth (Trypticase Soy Broth) herein referred to as Trypticase Soy Broth (see section 4.2).

7.10 #3 Broth (Nutrient Broth) herein referred to as Nutrient Broth (see section 4.2).

8.0 INSTRUMENT OR METHOD CALIBRATION: Not applicable

9.0 SAMPLE HANDLING AND STORAGE:

9.1 Refer to section 10.0, Procedure and Analysis, for storage conditions for test microbes.

10.0 PROCEDURE AND ANALYSIS:

10.1 *Pseudomonas aeruginosa, Staphylococcus aureus,* and *Bacillus subtilis*

10.1.1 Culture Initiation.

10.1.1.1 Every 12 months (or sooner if necessary) initiate new stock cultures from lyophilized cultures of *Pseudomonas aeruginosa* (ATCC 15442), *Staphylococcus aureus* (ATCC 6538), and *Bacillus subtilis* (ATCC 19659) from the American Type Culture Collection (ATCC) or other reputable supplier.

10.1.1.2 Open ampule of freeze dried organism as indicated by ATCC.

10.1.1.3 For *Staphylococcus aureus* and *Pseudomonas aeruginosa*, using a tube of Trypticase Soy Broth withdraw approximately 0.5 to 1.0 mL with a Pasteur or 1.0 mL pipette and rehydrate the pellet. For *Bacillus subtilis*, using a tube of Nutrient Broth withdraw approximately 0.5 to 1.0 mL with a Pasteur or 1.0 mL pipette and rehydrate the pellet.

10.1.1.4 Using several drops of the suspension, inoculate a tube of broth (Trypticase Soy Broth for *Staphylococcus aureus* and *Pseudomonas aeruginosa* and Nutrient Broth for *Bacillus subtilis*) indicated as "TUBE A," (see Attachment 1).

10.1.1.5 Incubate broth culture (TUBE A) at 37±1ºC for 24±2 hours for *Staphylococcus aureus* and *Pseudomonas aeruginosa*. Incubate broth culture (TUBE A) at 30±1ºC for 24±2 hours for *Bacillus subtilis*.

10.1.1.6 Record all manipulations on the Organism Culture Tracking Form.

10.1.2 Culture Identification and Quality Control.

10.1.2.1 Initial confirmation testing for quality control (QC) and the generation of the first series of stock cultures will be performed concurrently using the 24±2 hours old broth culture identified as TUBE A (see Attachment 1) from step 10.1.1.5.

10.1.2.2 Use the 24±2 hour TUBE A broth culture to perform a 1:10 dilution series in phosphate buffered dilution water out to 1×10^{-8}. Plate dilutions 1×10^{-6} to 1×10^{-8} on TSA by the spread plate method. It is not necessary to plate the dilutions in duplicate. Incubate the plates for 18-24 hour. Carefully examine the plates for uniform colony morphology on all plates. Look for colonies that do not match the typical colony morphology described in Table 1.

10.1.2.3 For *S. aureus* and *P. aeruginosa*, streak a loopful of broth from TUBE A onto both selective media (MSA and Cetrimide). Selective media is not used for *Bacillus subtilis*.

10.1.2.4 Incubate plates at 37±1ºC for 24±2 hours.

10.1.2.5 Following the incubation period, record the colony morphology on the TSA dilution plates and selective media plates (including the absence of growth) and stain reaction. See Table 1 for details on cell and colony morphology, colony characteristics on selective media, and stain reactions.

10.1.2.5.1 For *S. aureus*, note the organism's growth characteristics on MSA (colony size, color, texture, etc.) and Cetrimide (absence of growth). For *P. aeruginosa*, note the organism's growth characteristics on Cetrimide (colony size, color, texture, etc.) and MSA (absence of growth). Check for consistency with the genus and species of the organism to be tested (round, shiny, and yellow for *S. aureus* on MSA, and flat, greenish-yellow, and opaque for *P. aeruginosa* on Cetrimide).

10.1.2.5.2 For each organism, perform a Gram stain from growth taken from a TSA dilution plate that was diluted and plated directly from TUBE A (see Attachment 1). Perform the Gram stain according to the manufacturer's instructions. Observe the Gram reaction by using brightfield microscopy at 1000× magnification (oil immersion).

10.1.2.6 Vitek 32 System: Using growth from an 18-24 hour TSA dilution plate diluted and plated directly from TUBE A (see Attachment 1), perform the necessary tests for each organism as outlined in the PINSERT printout available from the Vitek 32 System and the instructions stated in the Vitek manual (see SOP QC-16, VITEK: Culture Identification Numbers).

10.1.2.7 Record all confirmation results on the Test Microbe Confirmation Sheet (see 16.3).

10.1.3 Generation of Stock Cultures.

10.1.3.1 Use the 24±2 hour TUBE A (see Attachment 1) broth culture discussed in 10.1.1.5 to initiate stock cultures.

10.1.3.2 For *S. aureus* and *B. subtilis,* streak six nutrient agar slants each. For *P. aeruginosa*, stab six cystine trypticase agar (CTA) tubes.

10.1.3.3 Incubate the *S. aureus* slants and *P. aeruginosa* stabs at 37±1ºC for 48±2 hours. Incubate the *B. subtilis* slants at 37±1ºC for 24±2 hours.

10.1.3.4 Following incubation, store the cultures at 2-5ºC for 30 days (31 days for the *B. subtilis* slants). These cultures are identified as the "stock cultures." Begin stock culture transfers (outlined in section 10.1.5 under Culture Maintenance) on the 28th day of the 30 day storage period. Repeat the cycle for a maximum of one year.

10.1.3.5 From a set of six stock cultures, one is used every 30 days for QC and to generate new stock cultures, four may be used per month (one/week) for generation of test cultures, (see SOP MB-05, Use Dilution Method; and SOP MB-06, Testing Spray Disinfectants) and one is a back-up tube.

10.1.4 Monthly QC of Stock Cultures.

10.1.4.1 Monthly QC of stock cultures may occur just prior to or concurrently with stock culture transfers. Use one refrigerated stock culture tube and streak a loopful on a plate of TSA. For *S. aureus* and *P. aeruginosa*, streak a loopful onto both selective media (MSA and Cetrimide), as noted in sections 10.1.2.3 through 10.1.2.5.

10.1.4.2 Incubate the plates at 37±1ºC for 24±2 hours (18-24 hours for use in the Vitek 32 System). Follow steps outlined in

section 10.1.2 to confirm the identity of the organism.

10.1.5 Culture Maintenance.

10.1.5.1 On the 28th day of the 30 day stock culture storage period, initiate stock culture transfers. Use the same refrigerated stock culture tube used for Monthly QC described in 10.1.4.1 to inoculate 6 new stock cultures tubes as outlined in 10.1.3.2.

10.1.5.2 Incubate the new stock cultures as indicated in 10.1.3.3.

10.1.5.3 Following the incubation period, store the stock cultures at 2-5ºC for 30 days (31 days for the ***B. subtilis*** slants).

10.2 *Mycobacterium bovis* (BCG)

10.2.1 Culture Initiation.

10.2.1.1 Obtain lyophilized cultures of ***M. bovis*** (BCG) from Organon Teknika.

10.2.1.2 Reconstitute the lyophilized culture with ~1 mL of sterile DI water. Inoculate two Middlebrook 7H9 (M7H9) agar plates by streaking for isolation.

10.2.1.3 Add ~0.2 mL of the rehydrated culture to each of 4 tubes of Modified Proskauer Beck broth (MPB).

10.2.1.4 Incubate the M7H9 agar plates and MPB broth tubes for 15 to 20 days at 37±1ºC or until there is sufficient growth. MPB broth tubes should be incubated in a slanted position.

10.2.2 Culture Identification and Confirmation.

10.2.2.1 Note the organisms' growth characteristics on the M7H9 agar plates (colony size, color, texture, etc.). Check for consistency with the genus and species of the organism (off-white to buff-colored, raised, and rough for ***M. bovis*** (BCG)) (see Table 1). ***M. bovis*** (BCG) is a slow growing organism. Colonies become visible on M7H9 agar

in approximately 14 days.

10.2.2.2 Following the incubation period, perform an acid fast stain on a smear of the *M. bovis* (BCG) culture taken from the MPB broth tube or from a M7H9 plate and observe using brightfield microscopy at 1000× magnification.

10.2.2.3 A description of the acid fast stain reaction and the colony morphology of *M. bovis* (BCG) can be found in Table 1.

10.2.2.4 Record all confirmation results on the Test Microbe Confirmation Sheet (see 16.3).

10.2.3 Generation of Stock Cultures and Stock Culture Maintenance.

10.2.3.1 Once the confirmation steps are completed and appropriate results are obtained for *M. bovis* (BCG), use the 15 to 20 day old MPB broth cultures to initiate stock cultures.

10.2.3.2 Streak M7H9 agar slants (stock slants) using the 1-4 tubes of MPB broth cultures of *M. bovis* (BCG). Based on anticipated use, 10-20 stock slants should be streaked.

10.2.3.3 Up to fourteen of the stock slants will potentially be used to generate cultures for testing each month (see SOP MB-06, Testing Spray Disinfectants and SOP MB-07, Confirmatory Tuberculocidal Method), and 2 will be used to initiate the next month's stock slants.

10.2.3.4 Incubate the new stock transfers for 15 to 20 days at 37±1ºC. Store at 2-5ºC.

10.2.4 QC of Stock Cultures.

10.2.4.1 Each month or 6 weeks (30-42 days), for quality control purposes, select one of the 2 stock slants used to generate the additional 10-20 M7H9 slants and streak a loopful of growth for isolation on a plate of M7H9 agar.

10.2.4.2 Incubate the plate for 21-25 days at 37±1ºC. Evaluate the colony morphology and perform an acid fast stain as

described in sections 10.2.2.2 through 10.2.2.4.

10.2.4.3 Acid fast stain reaction and colony morphology of *M. bovis* (BCG) can be found in Table 1. Record observations on the Test Microbe Confirmation Sheet (16.3).

10.2.5 Culture Maintenance.

10.2.5.1 Each month or 6 weeks (30-42 days), use 2 stock slants to generate an additional 10-20 M7H9 slants. Inoculate new M7H9 slants by streaking a loopful of *M. bovis* (BCG) growth from an established tube to each of the 10-20 tubes.

10.2.5.2 Incubate the stock culture slant at 37±1ºC for 15 to 20 days. Store at 2-5ºC.

Table 1. Typical Growth Characteristics of strains of *P. aeruginosa, S. aureus, B. subtilis,* and *M. bovis* (BCG) (see ref. 15.4, 15.5 and 15.6).

	*P. aeruginosa**	*S. aureus**	*M. bovis* (BCG)**	*B. subtilis**
Gram stain rxn***	(-)	(+)	weakly (+)	(+)
Acid Fast stain rxn	N/A	N/A	(+)	N/A
Typical Growth Characteristics on Solid Media				
Mannitol Salt	No Growth	circular, small, yellow colonies, agar turning fluorescent yellow	N/A	N/A
Cetrimide	circular, small, initially opaque, turning fluorescent green over time; agar fluorescent yellowish green	No Growth	N/A	N/A
Middlebrook 7H9	N/A	N/A	rough, raised, thick colonies with a nodular or wrinkled surface and an irregular thin margin, off-white to faint buff, or even yellow	N/A
TSA	flat, opaque to off-white, round spreading (1)	small, circular, yellow glistening	N/A	opaque, rough, dull, round, low convex colonies with irregular margins
Typical Microscopic Characteristics				
Cell dimensions	0.5-1.0 μm in diameter by 1.5-5.0 μm in length*	0.5-1.5 μm in diameter*	0.3-0.6 μm in diameter by 1-4 μm in length**	0.8-1.0 µm in diameter by 3.5-5.0 μm in length*
Cell appearance	straight or slightly curved rods, single polar flagella, rods formed in chains	spherical, occurring singly, in pairs and tetrads, sometimes forming irregular clusters	rods, straight or slightly curved, occurring singly and in occasional threads	rods, singly or in pairs, motile by peritrichous flagella, production of central spores

*After 24±2 hours
**After 15-20 days
***rxn = reaction
(1) Plates from dilution plating. The agar plate may show three different colony types: a) circular, undulate edge, convex, rough and opaque; b) circular, entire edge, convex, smooth and translucent; c) irregular, undulate edge, convex, rough, spreading, and translucent. Colony c) reverts to colony type a) after 24 hour incubation. Pyocyanin is not produced.

10.3 Supply Control Number

10.3.1 All cultures are given a supply control number upon receipt (see SOP QC-09, Control Numbers).

10.3.2 The supply control number will consist of the date received (R) and the date the ampule expires (E).

For example: For a *S. aureus* dehydrated ampule received on 06-21-05 and expiring on 10-07-06, the supply control number would be R062105-E100706. For a *M. bovis* (BCG) dehydrated ampule received on 07-24-05 and expiring on 05-04-06, the supply control number would be R072405E050406.

10.4 Microbe Received and Microbe Expiration Number (MRME).

10.4.1 The MRME number will consist of the date received (MR) and the date the reconstituted microbe expires (ME). The ME number is not required for *M. bovis* (BCG) because the culture does not expire.

10.4.1.1 *P. aeruginosa*, *S. aureus*, and *B. subtilis*. A culture received on 06-21-05 and reconstituted on 07-28-05 would receive a culture notation of MR062105ME072805, where "MR" represents microbe received and "ME" represents the date the reconstituted microbe expires.

10.4.1.2 Once reconstituted, *P. aeruginosa*, *S. aureus*, and *B. subtilis* can only be transferred for a period of one year.

10.4.1.3 Once expired, the stock cultures must be autoclaved and discarded and a new culture initiated from a new lyophilized lot from ATCC.

10.4.1.4 *M. bovis* (BCG). *M. bovis* (BCG) is not required to be replaced annually. Therefore, the culture notation will only consist of the MR number.

10.4.1.5 Continuous transfers of *M. bovis* (BCG) may be made unless the organism has been compromised.

10.4.2 Additional Culture Notation. The MRME culture notation will have a suffix as follows: for *Staphylococcus* the suffix will be S; for *Pseudomonas* the suffix will be P; for *Bacillus* the suffix will be B; for *Mycobacterium* the suffix will be M.

Thus, the final culture notation after reconstitution for *Staphylococcus*, for example, would be MRXXXXXXMEXXXXXX-S. The culture notation for *M. bovis* (BCG) would be MRXXXXXX-M.

10.5 Culture Transfer Notation of Test Microbes.

10.5.1 See footnotes for Organism Culture Tracking Form for transfer notations (see 16.1). For example, *Staphylococcus* test culture notation would be MRXXXXXXMEXXXXXX-S-06-04TC, where 06 is the monthly transfer (number of months since the month of initiation) and 04 is the 4th daily transfer; TC is applied to indicate a 48 hour test culture.

10.5.2 See footnotes for Organism Culture Tracking Form for *M. bovis* (BCG) transfer notations (see 16.2). For *Mycobacterium*, test culture notation would be MRXXXXXX-M-11-03, where 11 represents the month of transfer (the month of the year) and 03 represents the 3rd week of the month for that transfer. TC is applied to identify the test culture. Since test culture transfers typically occur every Monday (or Tuesday), the weeks of each month are numbered consecutively starting with the 1st Monday of the month (as 01) and ending with the last Monday of the month (depending on the # of Mondays in the month, as either 04 or 05).

11.0 DATA ANALYSIS/CALCULATIONS: None

12.0 DATA MANAGEMENT/RECORDS MANAGEMENT:

12.1 Data will be recorded promptly, legibly, and in indelible ink on the Test Microbe Conformation Sheet and the Organism Culture Tracking Form. Completed forms are archived in notebooks kept in secured file cabinets in the file room D217. Only authorized personnel have access to the secured files. Archived data is subject to OPP's official retention schedule contained in SOP ADM-03, Records and Archives.

13.0 QUALITY CONTROL:

13.1 For quality control purposes, the required information is documented on the appropriate record form(s) (see 16.0).

14.0 NONCONFORMANCE AND CORRECTIVE ACTION:

14.1 If the results of quality control do not verify the identity of the test organism, then the culture is discarded and a new culture is initiated. New stock cultures are established as outlined in section 10.0 of this SOP.

15.0 REFERENCES:

15.1 bioMérieux S.A. 1995. Industrial Vitek Reference Manual No. 510713-1, Revision Date 07-1995. bioMérieux Vitek, Inc., Hazelwood, MO.

15.2 bioMérieux S.A. 1997. Analytical Profile Index Reference Book Number 20 090 (20 NE), 6th Edition. Marcy-l´Etoile, France.

15.3 bioMérieux S.A. 1997. Analytical Profile Index Reference Book Number 20 590 (Staph),4th Edition. Marcy-l´Etoile, France.

15.4 Holt, J., Krieg, N., Sneath, P., Staley, J. and Williams, S. eds. 1994. Bergey's Manual of Determinative Bacteriology, 9th Edition. Williams & Wilkins, Baltimore, MD.

15.5 Krieg, Noel R. and Holt, John G. 1984. Bergey's Manual of Systematic Bacteriology Volume 1. Williams & Wilkins, Baltimore, MD.

15.6 Sneath, P., Mair, N., Sharpe, M.E., and Holt, J. eds. 1986. Bergey's Manual of Systematic Bacteriology Volume 2. Williams & Wilkins, Baltimore, MD.

16.0 FORMS AND DATA SHEETS:

16.1 Organism Culture Tracking Form

16.2 Organism Culture Tracking Form for *Mycobacterium bovis* (BCG)

16.3 Test Microbe Confirmation Sheet

16.4 Attachment 1: Culture Initiation Flow Chart for *S. aureus*, *P. aeruginosa*, and *B. subtilis*

ORGANISM CULTURE TRACKING FORM

OPP Microbiology Laboratory

Organism:		Supply Control Number:	
Source and Strain no.:		Lot Number:	

Date	Time	Init.	Subculture Source	Transfer*		Media Inoculated (and # inoc.)	Media Prep No.	Incubation Conditions	Comments
				Monthly	Daily				

* "Monthly" indicates the monthly transfers for culture and "Daily" indicates a 24/48 hr serial transfer (added to control number)
NR = None Required
TC = Test Culture, applied after daily transfer number

ORGANISM CULTURE TRACKING FORM FOR *Mycobacterium bovis* (BCG)

OPP Microbiology Laboratory

Organism:	*Mycobacterium bovis* (BCG)	MR Number:	
Source and Strain no.:	Organon Teknika	Lot Number:	

Date	Time	Init.	Subculture Source	Transfer		Media Inoculated (and # inoc.)	Media Prep No.	Incubation Conditions	Comments
				Month*	Week**				

* Indicates the month of the year the culture was transferred; added to MR number
** Indicates the week of the month for the transfer (week 1-5); added to MR number
*** Weekly and TC cultures may be used on days 21-25
TC = Test Culture, applied after weekly transfer number
NR = None Required; N/A = Not Applicable; SC = Stock Culture; QC = Quality Control; d = days

TEST MICROBE CONFIRMATION SHEET (Quality Control)

OPP Microbiology Laboratory

Organism:		MRME*** Number:	
Source and Strain no.:		Notes:	

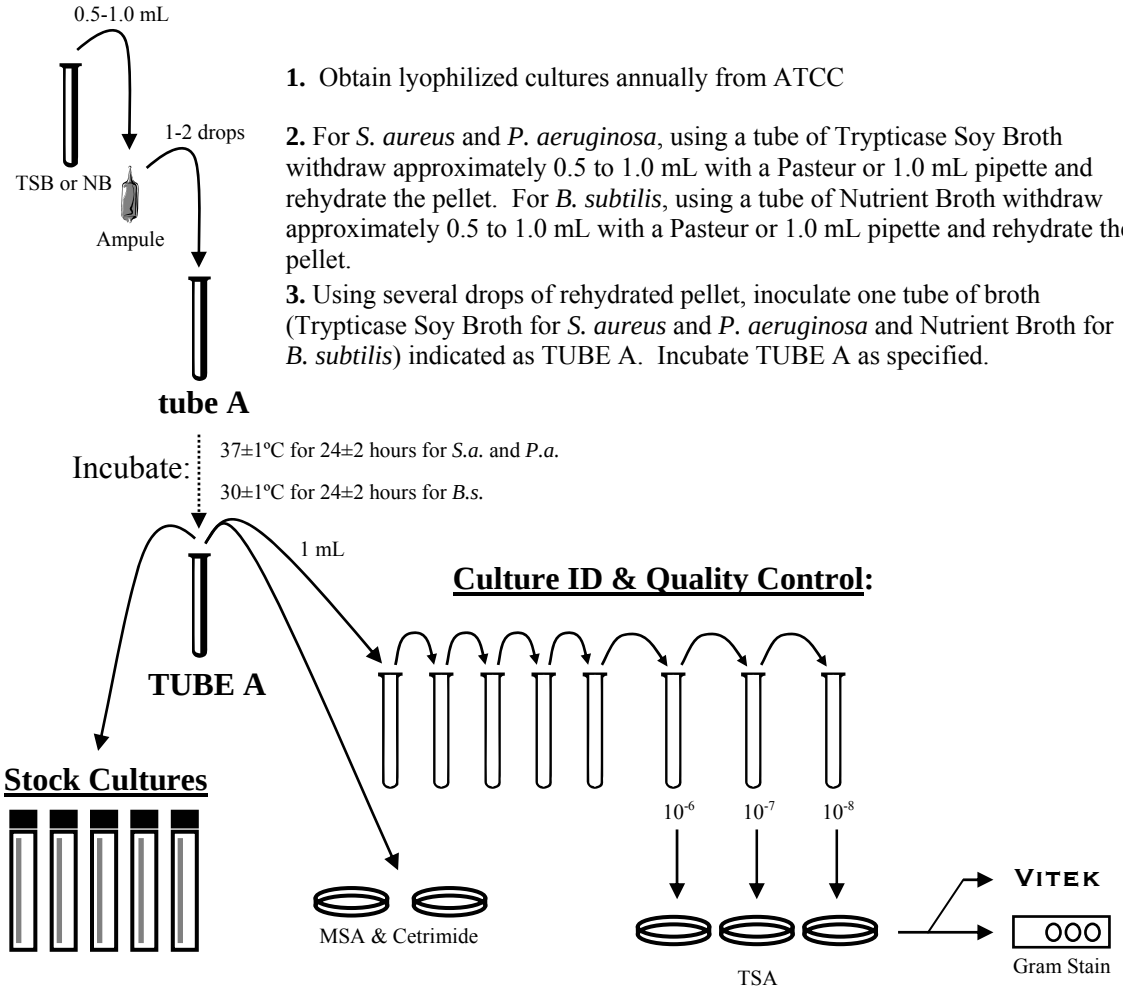
Source: Tube/Plate ID	Date/ Initials	Staining Results*	Media Information			Results		
			Name	Prep. No.	Inc. Time/ Temp.	Date/Initials	Colony Characteristics	API/Vitek Log #**

* Record Gram stain results or Acid Fast staining results, GPC = Gram Positive Cocci; GNR = Gram Negative Rods; AFR = Acid Fast Rods. GPR = Gram Positive Rods

** API profile number

*** MRME notation will be for all organisms except *M. bovis* (BCG) and all Vitek QC organisms. Use only MR notation for *M. bovis* (BCG) and the appropriate Vitek QC number assigned to each organism.

Attachment 1: Culture Initiation Flow Chart for *S. aureus*, *P. aeruginosa*, and *B. subtilis*



1. Obtain lyophilized cultures annually from ATCC

2. For *S. aureus* and *P. aeruginosa*, using a tube of Trypticase Soy Broth withdraw approximately 0.5 to 1.0 mL with a Pasteur or 1.0 mL pipette and rehydrate the pellet. For *B. subtilis*, using a tube of Nutrient Broth withdraw approximately 0.5 to 1.0 mL with a Pasteur or 1.0 mL pipette and rehydrate the pellet.

3. Using several drops of rehydrated pellet, inoculate one tube of broth (Trypticase Soy Broth for *S. aureus* and *P. aeruginosa* and Nutrient Broth for *B. subtilis*) indicated as TUBE A. Incubate TUBE A as specified.

4. <u>STOCK CULTURES</u> –

- **Using the 24±2 hour TUBE A broth culture:** initiate stock cultures. For *S. aureus* and *B. subtilis,* streak inoculate six nutrient agar slants. For *P. aeruginosa*, stab inoculate six cystine trypticase agar (CTA) tubes. Incubate the *S. aureus* slants and *P. aeruginosa* stabs at 37±1ºC for 48±2 hours. Incubate the *B. subtilis* slants at 37±1ºC for 24±2 hours. Record all manipulations on the Organism Culture Tracking Form.

5. <u>CULTURE ID & QUALITY CONTROL</u> –

- **Using the 24±2 hour TUBE A broth culture:** perform a 1:10 dilution series in phosphate buffered dilution water out to 1×10^{-8}. Plate dilutions 1×10^{-6} to 1×10^{-8} on TSA by the spread plate method. It is not necessary to plate the dilutions in duplicate. Incubate the plates for 18-24 hour. Use the plates for purity evaluation, Gram Stain, and Vitek analysis. Record results on the Test Microbe Confirmation Sheet.

- **Using the 24±2 hour TUBE A broth cultures:** for *S. aureus* and *P. aeruginosa*, streak a loopful onto both selective media (MSA and Cetrimide). Selective media is not used for *Bacillus subtilis*. Incubate plates at 37±1ºC for 24±2 hours. Record results on the Test Microbe Confirmation Sheet.